Scientific Bulletin. Series F. Biotechnologies, Vol. XVII, 2013
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

INDUCTION OF INDIRECT ORGANOGENESIS *IN VITRO* IN *RHODIOLA ROSEA*– AN IMPORTANT MEDICINAL PLANT

KrasimiraTASHEVA and Georgina KOSTURKOVA

Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria,

Corresponding author email: E-mail: krasitasheva@yahoo.com

Abstract

Rhodiola rosea L. is an endangered medicinal plant due to over harvesting in Bulgaria and in other European countries. The root and rhizomes are rich in pharmacologically and therapeutically active substances like flavonoids, phenolic acids that make the plant of commercial importance. The seed propagation in nature is very poor as far as only of 2 to 30 % of the seeds germinate depending on the ecologically geographic conditions. Thus, in vitro techniques are suitable for propagation of this species. In vitro propagation is possible by direct and indirect organogenesis. The latter is much more difficult in many species, including golden root. The present study aimed to understand the conditions, which provoke undifferentiated tissue initiation and development of shoots.

The effects of different plant growth regulators in various concentrations oncallus induction and indirect plant regeneration were investigated. Regenerative callus was received on Murashige and Skoog medium containing 2,4-Dichlorophenoxyacetic acid (0.1 mg l^{-1})within 28 days. The adventitious buds were differentiated when the calli were subcultured on Murashige and Skoog (1962) medium supplemented with 6-benzylaminopurine (1.0 mg l^{-1}) and indole-3-acetic acid (0.1 mg l^{-1}) within 4 - 5 weeks. Half-strength solid MS with indole-3-butyric acid (2.0 mg l^{-1})and IAA (0.2 mg l^{-1}) exhibited the best in vitro rooting.

These results contribute to the understanding of processes of growth and development in vitro of Rhodiola rosea and possibilities for selected of valuable clones and establishment of propagation schemes.

Keywords*: calli, golden root, indirect organogenesis, regeneration.*

INTRODUCTION

More than 2000 plant species in Europe are used for production of medical and nutraceutical herb preparations. The wild species represent 70 % from this production and only 30 % from the species are cultivated in the field. However, a significant number of the wild species are rare, disappeared and/or under protection (Varbanova, 2002; Evstatieva et al., 2007). One of the reasons is the increasing interest of pharmaceutical industry towards these plants and respectively more intensive use of their natural resources which are not unlimited. This raises the question of searching for and development of alternative methods friendly to the environment. (Tasheva and Kosturkova, 2013). Such ones are the biotechnological methods which are important for breeding, propagation and conservation of valuable medicinal plants.

Biotechnological techniques using cells, tissues, organs or whole organisms, growing and developing in *in vitro* conditions, are suitable for genetic manipulations (Khan et al., 2009), and to obtain valuable compounds (Rao andRavishankar, 2002).

During the last years, propagation of medicinal plants in *in vitro* conditions has gained increasing interest to the industry and has been more widely used for the necessities of the pharmaceutical industry. The way leading to production of great number of identical plants, what is a subject of clonal/micro-propagation, is one of the most preferable approaches from a commercial viewpoint. This approach could be used to create *ex situ* and *in vitro* collections, as well as for commercial propagation to obtain raw material for pharmacetical and cosmetic industries (Julsing et al., 2006). *In vitro*propagation allows obtaining of a large plant mass for a short period of time (Tripathi and Tripathi, 2003). Developed

micropropagation methods have been reported for many medicinal plants but this list is increasing (Tasheva and Kosturkova, 2013).

Experimental approaches used for *in vitro* propagation of medicinal plants could be broadly divided/classified in three categories: (1) Isolation of meristems and stem tips and stimulation of their growth which depends on different factors. The effect of plant regulators and their combinations to micropropagation of medicinal plants is reported by Makunga et al., 2003; Debnath, 2009; Keng et al., 2009; Yusuf et al., 2011.

(2) The second way includes induction of adventitious buds from leaves, stems and root segments or obtaining callus from these organs. The plant regeneration by organogenesis is well developed in some medicinal and aromatic plants, but it varies widely for different plant species, which requires an individual approach to study the conditions for regeneration, as well as determining the factors controlling growth and differentiation for each type of species (Patra et al. 1998).

(3) The third way is somatic embryogenesis, which is the theoretically most effective to obtain plants (Martin, 2004; Paramageetham et al., 2004; Fiuk and Rybczynski, 2008, Robinson et al., 2009). To determinate, the relationship between different groups of phytoregulators is essential for these because success of tissue culture work depends on phytoregulators′ type and concentration. The process of differentiation of unorganized callus tissue, initiation of the buds and roots depends on the appropriate combination of auxins and cytokinins in the nutrient medium (Sagare et al., 2000). The influence of the cytokinins and auxins on *in vitro* cultures depends on the *in vitro* systems, plant species and in many cases even to the variety or/and ecotype. The natural and synthetic auxins and cytokinins induce typically physiological responses in plants. The specificity of the investigated object from one side and endogenous phytohormones from other side determine the effects of the exogenously applied phytoregulators.

Rhodiola rosea is a medicinal plant under protection in Bulgaria and other European countries. The extract from root and rhizomes has a number of applications. It has adaptogenic, antitoxic and antihypoxic action, and increases resistance to infection diseases. Due to the low toxicity and the absence of side effects of its extract, golden root is widely used in food and cosmetic industry. In Bulgaria, there are no systematic and comprehensive studies of this endangered species and data on the application of modern biotechnological and phytochemical approaches. Creating integrated technologies for breeding, preservation and cultivation of this valuable species is a major premise for its cultivation in different mountain regions in Bulgaria.

The few biotechnologically investigations concerning species from *Rhodiola* genus refer particularly to the process of indirect organogenesis. Callus induction followed by plant regeneration of *Rh. coccinea, Rh. sachalinesis, Rh. rosea* were studied by some authors (Kirichenko et al., 1994; Furmanova et al., 1995; Ishmuratova, 1998; Yin et al., 2004; Sha Hongetal., 2008; Liu Jianfeng et el., 2007; Liu Jian-feng et al., 2009).

The present study aimed to understand the conditions which provoke undifferentiated tissue initiation and development of buds and shoots investigating the effects of different plant growth regulators in various concentrations oncallus induction and indirect plant regeneration.

MATERIALS AND METHODS

Plant material. Explants isolated from *in vitro* propagated plants, obtained in our previous work were used in these experiments (Tasheva and Kosturkova, 2010a, b).

Culture media.

Nutrient media composition for callus induction was Murashige and Skoog (1962) medium containing zeatin 2.0 mg/l (Trans form), IAA 0.2 mg/l, casein hydrolisate 1000 mg/l, sucrose 3%, and agar-agar 0.6% (Tasheva and Kosturkova. 2010a, b).

Nutrient medium composition for induction of organogenic callus designated as mediumDwas basic Murashige and Skoog (1962) nutrient medium enriched with 0.1 mg/l 2,4-D, 3% sucrose and 0.6 % agar-agar.

Nutrient medium composition for bud formation was Murashige and Skoog (1962) nutrient medium containing BAP 1.0 mg/l, IAA

0.1 mg/l, sucrose 3% and agar-agar 0.6% (designated as BA medium).

Nutrient medium composition for shoot formation and plant was Murashige and Skoog (1962) medium containing zeatin 2.0 mg/l (mixed isomers), IAA 0.2 mg/l, casein hydrolisate 400 mg/l, sucrose 3%, and agar-agar 0.6% (Tasheva and Kosturkova. 2010a, b). The pH of all culture media was adjusted to 5.7 – 5.8 prior and autoclaved at 1.1 kg.cm^{-2}, 121°C for 20 min.

Culture conditions. The calli explants were cultivated in test tubes or Petri dishes.

The frequency of regenerants induction was evaluatedperiodically with an interval of 28 days. Necrotic tissue was removed during each sub-cultivation. The average number and size of the buds, shoots and regenerants from one explant were scored. Obtained regenerants were separated from the callus and cultivated on media for multiplication followed by rooting.

The cultures were cultivated (induced and maintained) in a growth room with artificial illumination (fluorescent lamps) under a 16 h photoperiod at temperature of 21 - 23°C and light intensity 20 μM m^{-2} s^{-1} for callus initiation and maintenance and 40 μM m^{-2} s^{-1} for organogenesis induction and bud/shoot formation. The regenerants which had formed roots *in vitro* were transferred to small pots containing soil, peat and perlite.

RESULTS AND DISCUSSIONS

Callus induction. Callus like structures or callus in the shoot basis was formed using the developed scheme for micropropagation (Tasheva and Kosturkova. 2010a, b). About 0.6 – 1.0 % from the plants formed non differentiated tissue. In most of the cases (about 85 – 90%) callus was solid, grainy, with green-brown color (Figure 1 and Figure 2) and was considered appropriate for the present experiments.

Auxins, cytokinin and auxin/cytokinin interactions are usually considered the most important factors for regulating growth and organized development in plant tissue and organ culture. Plants generally require these two classes of growth regulators. The formation of calli may be due to accumulation of the auxins in the main cutting edges which stimulates cell proliferation especially in the presence of cytokinins (Martin 2000). The application of plant growth regulators tested concentrations significantly affects the growth and the development of callus and its differentiation.

Figure 1 Regenerated plants and calli formation on MS medium containing 2.0 mg/l zeatin, 0.2 mg/l IAA and 1000 mg/l casein hydrolisate

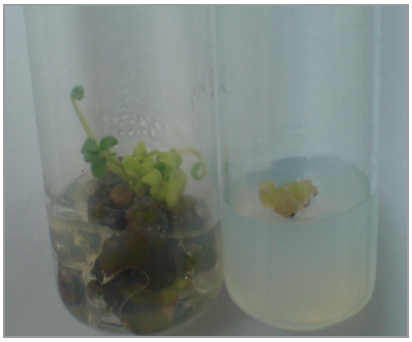

Figure 2. Formed and isolated callus tissue

Induction of organogenic callus and proliferation. Obtained callus when transferred to fresh nutrient medium D **(**containing MS salts and vitamins, 0.1 mg/l 2,4-D, 3% sucrose and 0.6% agar-agar) formed buds for 2 passages with 28 days duration of every passage (Figure 2 and Figure 3). Cultivation of the calli on this media for a one passage did not bring to revealing of organogenic capacity of calli.

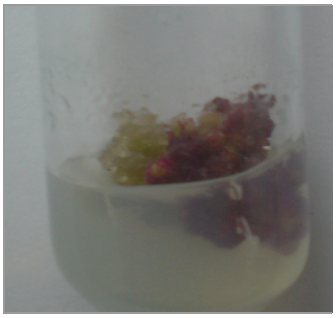

Figure 3. Isolated calli and cultivated on MS medium containing 0.1 mg/l 2,4-D

Bud and shoot induction. Bud formation was stimulated when the calli were cultivated on BA medium (MS nutrient medium containing BAP 1.0 mg/l, IAA 0.1 mg/l, sucrose 3% and agar-agar 0.6% (Figure 4).Thus, in the present study it was found that the BAP and BA concentration with auxin (IAA and BA) induced the organogenesis.

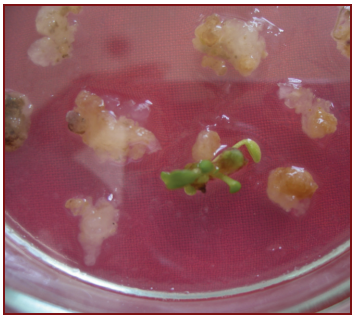

Figure 4. Indirect organogenesis and bud formation on MS medium containing BAP 1.0 mg/l, IAA 0.1 mg/l

Multiplication of the obtained regenerants. The frequency of regenerated plants from callus cultures was very low not exceeding 5 – 6 %. However, it was possible obtained mini plants to be multiplied on the same medium (Figure 5) or on a medium containing zeatin (cis/trans = 82/18% and IAA (for a short period of time) (Figure 6).

Studied concentrations of growth regulators and their combination, stimulated cell division in the tissues, buds growth and appear of the first leaf. Received healthy plants are ready for *in vitro* cultivation. These preliminary results show that BAP supported indirect organogenesis process by stimulating the cells to reach the competent phase for cell division.

Figure 5. Isolated buds and shoot formation on MS medium containing BAP 1 mg/l and IAA 0.1 mg/l

A high concentration of cytokinin inhibits callus formation and increases buds induction. The combinationof cytokinin and auxin has a positive effect on cell division and differentiation.

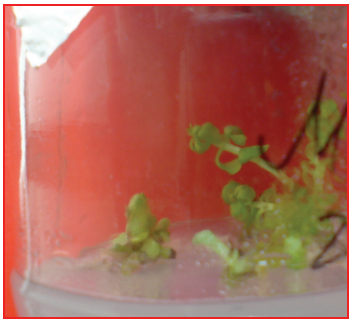

Figure 6. Plant multiplication on MS medium containing BAP 1 mg/l and IAA 0.1 mg/l

Callus is dedifferentiated and unorganized mass of parenchyma cells formed by the proliferation of parent tissue. Callus tissue is a good source of genetic variability and adventitious shoot formation. Callus induction is a prerequisite for

adventitious shoot formation and also for the other *in vitro* genetic improvement including induction of somaclonal variations and embryoids.

Difficulties in working with callus cultures of different *Rhodiola* species were reported by other authors, too. Kirichenko et al. (1994) and Yin et al. (2004) studied the possibilities for callusogenesis, organogenesis and regeneration form leave explants of *Rh. rosea*. Furmanova et al. (1995) examined the effects of the different nutrient media at the regeneration and callus formation capability of *Rh. rosea* and study biologically active substances in the roots in wild species. Ishmuratova (1998) demonstrated that the ecotype is also a factor influencing the processes of effective callusogenesis and organogenesis. The latest capability is studies in seeds and rhizomes from three ecotypes Rhodiola growing in high Altai using MS nutrient medium containing different phytoregulators – BA, IAA, NAA, IBA and 2,4-D. The author specified that the choice of the optimal nutrient medium (MS containing BAP (0.2 mg/l) and IAA (0.1 mg/l) for explant development is depended on ecotype.

Callus induction followed by plant regeneration of *Rhodiola coccinea* was studied byof ShaHongetal. (2008). The authors obtained three-type calli and noted that only one type of them was embryogenic. Similarly to our observations the role of BA and IAA was crucial. The embryogenic callus was obtained on MS nutrient medium containing BA (0.5 mg/l) and IAA (2.0 mg/l) and is suitable for regeneration. Callus induction (88.33 %) in different colors (yellow, green and red) was obtained of *Rhodiola sachalinsis* using leaf explants and cultivated on MS medium additionally with BA (2.0 mg/l) and NAA (0.5 mg/l) (Liu Jianfeng et el., 2007). They concluded that only green callus is able to regeneration on MS media containing BA (1.0 mg/l) and NAA (0.1 mg/l) with comparatively low percent (21.33 %) after long period of cultivation 50 days. The necessity for a long cultivation period was noted in our experiments, too. Regenerants were successfully rooted on½ MS. In the same species *Rhodiola* LiuJian-feng et al. (2009) obtained protoplast cultures from leaves of *in vitro* propagated plants, followed by callus induction and bud formation. The protoplasts formed callus on MS medium containing 2,4-D (1.0 mg/l), zeatin (0.5 mg/l), 0.5 M/l manitol and500 mg/l casein hydrolysate for 40-days period of cultivation. The callus formed adventitious buds on MS nutrient medium added with BA and NAA (1.0 mg/l and 0.1 mg/l, respectively). The buds grew and induced roots on ½MS for 30 days.

***In vitro* rooting.** The process of mini plants rooting is an important for their next step to adaptation in *ex vitro* conditions (Hazarika, 2003). The processes of rooting have specific requirements and rhyzogenesis was not obtained in all of the cited references. The rooting process on MS media without auxins has been studied in many plant species, such as *Echinaceapurpurea* (Korachetal., 2002), *Carlinaacaulis* (Trejgelletal., 2009). The frequency of the induced roots on medium without auxins may be due to the presence of endogenous auxins in regenerated buds/shoots (Minocha, 1987). Root induction of the growing *in vitro* shoots was achieved by adding auxins in nutrient media or on media without regulators which depends on species genotype (Rout et al., 1989). Different species had different potential to form roots and the optimal conditions are determined empirically. Moderate to high concentration of all auxins in the media inhibited root growth.

Figure 7. Root induction on MS medium containing BAP 1.0 mg/l and IAA 0.1 mg/l after 36 days cultivation.

The presence of auxin for the rhizogenesis process is necessary for many medicinal species. The concentration of IBA, for example, plays key role for stimulation of root formation for a number of plants as *Centaurea rupestris* (Perica, 2003), *Wedelia chinensis*

(Kameri et al., 2005), *Emilia zeylanica* (Robinson et al., 2006). Reducing the amount of salts in nutrient media for rhizogenesis had different effects to root formation and depend on the species. In many medicinal plants, rooting is more successful when the macro/micro salts (sometimes vitamins) are reduced twice or more *Saussurea obvallata* (Joshi and Dhar, 2003); *Ensete ventricosum* (Birmeta and Welander, 2004); *Ecliptaalba* (Baskaran and Jayabalan, 2005; *Carlina acaulis* (Trejgell et al., 2009), and sucrose is reduced to 2.0, 1.0 or 0.5%.

Figure 8. Roots formed on MS medium containing 2.0 mg/lIBA, 0.2 mg/lIAAand 0.4 mg/lGA_3

Figure 9. Propagation on MS medium containing zeatin (cis-trans form).

When regenerated shoots attained a height of 1.0-1.3 cm they were excised and transferred to MS medium for root induction. In our experiments rooting was observed on the same medium used for multiplication (Figure 7). Root formation needed about 28-36 days period of time, which is longer period in comparison with the use of half strength MS nutrient medium containing 2.0 mg/lIBA, 0.2 mg/lIAAand 0.4 mg/lGA_3, reported in our previous papers (Tasheva and Kosturkova, 2010 a,b) (Figure 8 and 9).

Adaptation *ex vitro*. Determination of the optimal conditions for adaptation of the invitro obtained plants in *exvitro* condition after the stage of rooting is a significant step for completed propagated scheme report. Adaptation of the regenerants in external conditions is the one critical stage of micripropagation. Adaptation of the plants obtained from this process of indirect regeneration was performed by the use of the scheme mentioned in our previous studies (Tasheva and Kosturkova, 2010a,b)

A model protocol for indirect organogenesis:

1. Callus induction on nutrient media containing zeatin 2.0 mg/l, IAA 0.2 mg/l and casein hydrolisate 1000 mg/l (MSZ1 medium). The callus was isolated and cutting on the explants with 0.5/1.0 cm in size.
2. Cultivation of the calli on MS media containing 0.1 mg/l 2,4-D (D medium) for 28 days, 22^{o}C and 16/ 8photoperiod - 2 passages
3. Buds regeneration on MS media containing BAP 1.0 mg/l and IAA 0.1 mg/l for 28 days, 22^{o}C and 16/ 8photoperiod
4. Propagation of plants regenerants on MS nutrient medium containing1.0 mg/l BAP and 0.1 mg/l IAA (BA medium) or on MS containing 1.0 mg/l zeatin and 0.1 mg/l IAA for 28 days, 22^{o}C and 16/ 8photoperiod
5. Rooted of the regenerants on ½MS media containing 2.0 mg/lIBA, 0.2 mg/lIAAand 0.4 mg/lGA_3(½ MS rooting medium**)** for 28 days, 22^{o}C and 16/ 8photoperiod.
6. Adaptation in *exvitro* conditions (Tashevaand Kosturkova, 2010 a,b)

CONCLUSIONS

As a result of our experiments the following scheme of consecutive culture media for indirect organogenesis and regeneration could be proposed: **MSZ2 →D→D→BA→½ MS.**

Plants produced in this study appeared normal without observed morphological or phenotypical abnormalities and successfully developed in pots. The present system could be used as an alternative one for multiplication of selected valuable clones of this important

medicinal plant. This gives possibility to enrich the spectrum in a population including by the methods of gene transfer.

ACKNOWLEDGEMENTS

Research was supported by the grant №BG051PO001-3.3.06-0025, financed by the European Social Fund and Operational Programme Human Resources Development (2007 – 2013) and co-financed by Bulgarian Ministry of Education, Youth and Science and by the National Science Fund of Bulgaria, Project for Junior Scientists DMU 03/55 (leader Dr. K. Tasheva).
Research is a part of COST Action FA0903 activities.

REFERENCES

Baskaran P, Jayabalan N., 2005. An efficient micropropagation system for *Eclipaalba*– a valuable medicinal herb. *In Vitro* Cellular and Developmental Biology –Plant, 41, p. 532–539.

BirmetaG., WelanderM., 2004. Efficient micropropagation of *Ensete ventricosum*applying meristem wounding: a three-step protocol. Plant Cell Reports, 23, p. 277–283.

Debnath Samir C., 2009. Zeatin and TDZ-induced Shoot Proliferation and Use of Bioreactor in Clonal Propagation of Medicinal Herb, Roseroot (*Rhodiola rosea* L). J. Plant Biochemistry & Biotechnology, 18(2), p. 245-248

Evstatieva L., R.Hardalova & K.Stoyanova, 2007. Medicinal plants in Bulgaria: diversity, legislation, conservation and trade. PhytologiaBalcanica, 13 (3), p. 415–427.

Fiuk A., Rybczyn´ski J.J., 2008. Factors influencing efficiency of somatic embryogenesis of *Gentiana kurroo* (Royle) cell suspension. Plant Biotechnol Rep., 2(1), p. 33–39

Furmanowa M, Oledzka H, Michalska M, Sokolnicka I & Radomska D., 1995.*Rhodiola rosea* L. (Roseroot): *In vitro* regeneration and the biological acivity of roots. Biotechnology in Agriculture and Forestry Vol. 33, Medicinal and Aromatic Plants VIII: p. 412-426.

Hazarika BN., 2003. Acclimatization of tissue-cultured plants. Current Science, 85, p.1704–1712.

Ishmuratova M.M., 1998. Clonal micropropagation of *Rhodiola rosea* L. and *R. iremelica* Boriss. *in vitro",* Plant resources, vol. 34, no. 1, pp. 12-23 (in Russian)

Joshi M, Dhar U., 2003. *In vitro* propagation of *Saussurea obvallata* (DC.) Edgew. –an endangered ethnoreligious medicinal herb of Himalaya. Plant Cell Reports,21, p. 933–939

Julsing K.M., Wim J.Quax, Kayser O., 2006. The Engineering of Medicinal Plants: Prospects and Limitations of Medicinal Plant Biotechnology. In. Medicinal Plant Biotechnology: From Basic Research to Industrial Applications Oliver Kayser(Editor), WimJ.Quax(Editor). p. 618.

Kameri M, Shashidhara S, Rajasekaran PE, 2005. *In vitro* multiplication of *Wedeliachinensis* (Osbeck) Merr. Plant cell biotechnology and molecular biology, 6, p. 147-150.

Keng ChL, Yee LS, Pin PL., 2009. Micropropagation of *Gynura procumbens* (Lour.) Merr. an important medicinal plant. Journal of Medicinal Plants Research, v. 3(3), p. 105-111

Khan Mohamed Yassen, Saleh Aliabbas, Vimal Kumar and Shalini Rajkumar, 2009. Resent advances in medcinal plant biotechnology. Indian Journal of Biotechnology, vol. 8, p. 9 – 22.

Kirichenko E. B., Rudenko S. S., Baglaj B. M., Masikevich U. G., 1994. Leaf culture from *invitro* propagated *Rhodiola rosea*. Bulletin GBS, RAN, vol. 169, pp. 50-54 (in Russian)

Korach A, Juliani HR, Kapteyn J, And Simon JE., 2002. *In vitro* regeneration of*Echinacea purpurea* from leaf explants. Plant Cell, Tissue and Organ Culture, 69, p. 79–83.

Liu Jianfeng, Yan Xiufeng, Li Xia, Cheng Yunqing, 2007. Callus induction and plant regeneration from leaf explants of *Rhodiola sachalinesis* A. Bor. Journal of Northeast Forestry University, 02, doi: CNKI:ISSN:1000-5382.0.2007-02-012

Liu Jian-feng, Cheng Yun-qing, Chen Zhi-wen, 2009. Protoplast isolation and plant regeneration from leaves of *Rhodiola sachalinsis*. Chinese Traditional and Herbal Drugs, 07, p. 2010 - 2014 (in Chinese); doi: CNKI:SUN:ZCYO.0.2009-07-043

Makunga NP, Jager AK, Van Staden J., 2003. Micropropagation of *Thapsia garganica* -a medicinal plant. Plant Cell Rep., v. 21(10), p. 967-973.

Martin, K.P., 2000. Rapid Propagation of *Holostemma ada-kodien* Schult., A Rare Medicinal Plant, through Axillary Bud Multiplication and Indirect Organogenesis. Plant Cell Rep., 21, 112–117

Martin K.P., 2004. Plant Regeneration through Somatic Embryogenesis in Medicinally Important *Centella asiatica* L. *In Vitro* Cellular & Developmental Biology.Plant, v. 40 (6), p. 586-591

Minocha SC., 1987. Plant growth regulators and morphogenesis in cell and tissueculture of forest trees. In: BongaJM, Durjan DJ [eds.], MartinusNijhoff Publishers, Dordrecht. Cell and Tissue Culture inForestry, vol. 1, p. 50–66.

Patra A, Rai B, Rout GR, Das P., 1998. Successful plant regeneration from callus cultures of *Centella asiatica* (Linn.) Urban. Plant Growth Regulation, v. 24, p. 13–6.

Paramageetham Ch., Prasad Babu G., Rao J.V.S., 2004. Somatic embryogenesis in *Centella asiatica* L. an important medicinal and neutraceutical plant of India Plant Cell, Tissue and Organ Culture, 79(1), p. 19-24

Perica U. M., 2003. *InVitro* Propagation Of *Centaurea Rupestris*L. ActaBiologicaCracoviensiaSeriesBotanica, 45(2), p. 127–130.

Rao Ramachandra S and G.A.Ravinshankar, 2002. Plant cell cultures: Chemical factories of secondary metabolites. Biotechnology Advances, v. 20, p. 101–153.

Robinson J.P., J.S. Britto, J. Sebastinraj, V.D. Kumar1 And S.S.Kumar., 2006. Micropropagation of Emiliazeylanica C.B.Clarke by using explants of inflorescencerachis. Journal of Biological Research, 5, p.109 – 113

Rout G.R., Debata B.K. and Das P., 1989. *In vitro* mass-scale propagation of *Rosa hybrida* cv. landora. Current Science, v. 8 (15), p. 876–878.

Robinson JPh, Britto S.J, Balakrishnan V., 2009. Regeneration of Plants Through Somatic Embryogenesis in *Emilia zeylanica* C. B. Clarke a Potential Medicinal Herb Botany Research International, v. 2 (1), p. 36-41.

Sagare AP, Lee YL, Lin TC, Chen CC, Tsay HS., 2000. Cytokinin-induced somatic embryogenesis and plant regeneration in *Corydalis yanhusuo* (Fumariaceae) – a medicinal plant. Plant Sci., v. 160(1), p. 139-147

Sha Hong, Tang Fang, GaoYan, Zhang Rui-lin, 2008. Callus induction and plant regeneration in *Rhodiola coccinea*. Journal of Xinjiang Agricultural University, 31(3), p. 58–61 DOI: CNKI: SUN:XJNY.0.2008-03-015 (in Chinese)

Tasheva K., and KosturkovaG., 2013. Environmental Biotechnology, Chapter 11. Role of Biotechnology for Protection of Endangered Medicinal Plants. , In Tech, p. 235 - 285

Tasheva K. and Kosturkova G., 2010a. Bulgarian golden root *in vitro* cultures for micropropagation and reintroduction. Central European Journal of Biology, v. 5(6), 2010, p. 853-863

Tasheva K. and Kosturkova G., 2010b. *Rhodiola roseain vitro* cultures peculiarities. Scientific publications - Biotechnology, (UASVM) p. 103 – 112.

Trejgell A, Dąbrowska G, Tretyn A., 2009. *In vitro* regeneration of *Carlina acaulis*subsp. simplex from seedling explants. Acta Physiologica Plantarum, 31, p. 445–453.

Tripathi L. and Tripathi J.N., 2003. Role of biotechnology in medicinal plants. Trop. J. Pharm. Res., 2 (2), p. 243 – 253.

Varabanova K., 2002. Medicinal and aromatic plant diversity in Bulgaria– protection, collection, study, use and conservation. Report of a working group on medicinal and aromatic plants. First Meeting, GozdMartuljek, Slovenia, 12 - 14 September, 2002, p. 27-32.

Yin W. B., Li W., Du G. S., Huang Q. N., 2004. Studies on tissue culture of Tibetan *Rhodiola rosea*. Acta Bot. Boreal. Occident. Sin., 24, p. 1506 – 1510.

Yusuf NA, Annuar S, Khalid N., 2011. Rapid micropropagation of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. (a valuable medicinal plant) from shoot bud explants. African Journal of Biotechnology, vol. 10(7), p. 1194-1199